

Rapid, Affordable, and Scalable SARS-CoV-2 Detection From Saliva

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Here we present an inexpensive, rapid, and robust reverse-transcription loop-mediated isothermal amplification (RT-LAMP)-based severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection method that is easily scalable, enabling point-of-care facilities and clinical labs to determine results from patients' saliva directly in 30 minutes for less than \$2 per reaction. The method uses a novel combination of widely available reagents that can be prepared in bulk, plated, and frozen and remain stable until samples are received. This innovation dramatically reduces preparation time, enabling high-throughput automation and testing with time to results (including setup) in less than 1 hour for 96 patient samples simultaneously when using a 384-well format. By using a dual reporter (phenol red pH indicator for end-point detection and SYTO-9 fluorescent dye for real time), the assay also provides internal validation of results and redundancy in the event of an instrument malfunction.

KEY WORDS: RT-LAMP, Fluorescent LAMP, saliva detection, extraction-free detection

INTRODUCTION

Since early 2020, the world has been overtaken by the coronavirus diseases 2019 pandemic caused by infections of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). There have been many efforts by various groups around the globe to develop molecular and antigen-based assays for detection and surveillance of this infection. Early efforts using quantitative reverse transcription polymerase chain reaction (RT-qPCR)-based^{1,2} detection of nasopharyngeal swabs from individuals were quickly hampered by disruption of supply chains for reagents and consumables and insufficient testing sites across the world. These challenges led scientists to look for alternative methods for detection using a wide variety of biological samples that included oral/nasal swabs, mouth gargles,³ saliva,^{2,4–6} and urine.^{7,8} The detection methodologies also included non-RT-qPCR methods, such as reverse-transcription loop-mediated isothermal amplification (RT-LAMP^{2,9–11}), clustered regularly interspaced short palindromic repeats-based^{12–14} reverse-transcription recombinase polymerase amplification,¹⁵ or reverse-transcription recombinase-aided amplification,¹⁶ with their outputs coupled with fluorescent or colorimetric reporters as well as lateral flow strip platforms to facilitate readout processes. Some of the popular methods still included the requirement to isolate the virus from the sample, and this was a cause of concern again because of the extra steps and consumables required. The requirements for expensive instrumentation, reagents, and time prevents some of these

assays from being widely deployable especially to low-resource settings. Here we describe our modifications of the RT-LAMP assay using saliva, which is inexpensive, quick, and scalable for various resource settings and does not require RNA extraction.

During our protocol development, we noticed that the standard loop-mediated isothermal amplification (LAMP) assay protocol tended to generate false-positive reactions (albeit at later time points), and we questioned whether inclusion of the loop primers were necessary. Previous reports from other groups using LAMP have suggested that the loop primers could result in reproducibility issues with that protocol. Our analysis suggested that although exclusion of loop primers delayed the reactions, we could detect amplification reliably by omitting just the loop forward (LF) primer from the master mix. This observation resulted in our recipe with just 5 of the 6 commonly used primers for each gene in the LAMP protocol. Our assays included primer sets for the E and the N genes and is called EN* primer mix (**Fig. 1**).

METHODS AND MATERIALS

The assay is a modified version of the colorimetric RT-LAMP protocol developed by Tanner *et al.* at New England Biolabs (NEB),^{2,4,17–19} which includes guanidine hydrochloride for enhanced sensitivity and specificity. Further modifications included the addition of Antarctic thermolabile uracil deglycosylase (UDG) to reduce carryover contamination, thereby reducing false positives, and the inclusion of RNaseOUT recombinant RNase inhibitor or polyvinylsulfonic acid (PVSA) for improved sample stability.^{20,21} The saliva sample preparation method is modified from Rabe and Cepko's protocol.²²

The following reagents used in assay development were purchased from NEB (Ipswich, MA): WarmStart Colorimetric LAMP 2X Master Mix (DNA and RNA; M1800L), Warm-

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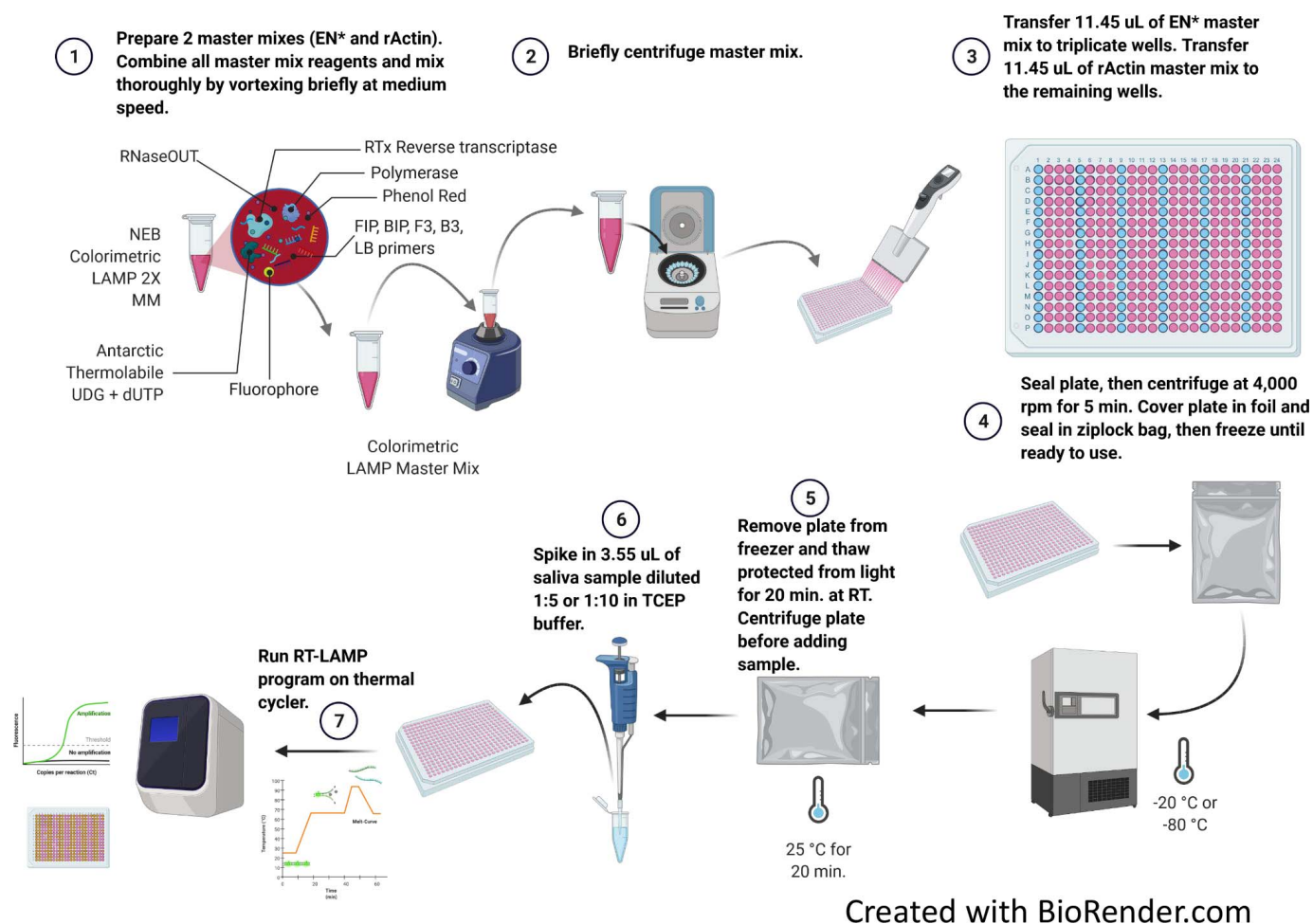


FIGURE 1

Schematic of dual-assay workflow. RT, room temperature; FIP, forward inner primer; BIP, backward inner primer; F3, forward outer primer; B3, backward outer primer; LB, loop backward primer.

Start LAMP Kit (DNA and RNA; E1700L), Antarctic Thermolabile UDG (M0372L), and dUTP Solution (N0459S). All primers used for early assay development were standard-desalted oligos synthesized by Integrated DNA Technologies (Coralville, IA). Assay optimization was performed using high-performance liquid chromatography-purified forward and backward inner primers (FIP and BIP) for SARS-CoV-2 E1 and N2 genes synthesized by LGC Bioscience Technologies (Hoddesdon, UK). Internal control primers for rActin were from NEB SARS-CoV-2 Rapid Colorimetric LAMP assay kit (catalog no. E2019S). Guanidine hydrochloride (Millipore CAS 5010; MilliporeSigma, Burlington, MA) and tris (2-carboxyethyl) phosphine (TCEP; Sigma-Aldrich C4706) were generously donated by Dr Qishan Lin of the RNA Epi-transcriptomics and Proteomics Resource, State University of New York Albany. Other reagents were purchased from Thermo Fisher Scientific and MilliporeSigma: Invitrogen UltraPure 0.5 M EDTA, pH 8.0 (catalog no. 15575020), Invitrogen RNaseOUT Recombinant RNase Inhibitor (catalog no. 10777019), Invitrogen SUPERase-In (catalog no. AM2696), Sigma Aldrich polyvinylsulfonic acid, sodium salt

solution 30% wt in H₂O (catalog no. 278424), and VWR Nuclease-Free Water (not diethylpyrocarbonate-treated; catalog no. 02-0201-1000). Positive controls used for assay validation included synthetic control RNA from Twist Bioscience (SARS-CoV-2 Control 1 MT007544.1, catalog no. 102019) and these BEI Resources: genomic RNA from SARS-CoV-2 isolate USA-CA3/2020 (NR-52507), SARS-CoV-2 isolate USA-WA1/2020 heat-inactivated cell lysate (NR-52286), and SARS-CoV-2 isolate USA-WA1/2020 γ -irradiated cell lysate (NR-52287).

Reaction Preparation

All reagents were thawed at room temperature and vortexed gently but thoroughly 10 seconds at medium speed and then quickly spun down and placed (except NEB Colorimetric LAMP 2X Master Mix and NEB 50X fluorescent dye) on ice until ready to assemble. The 50X fluorescent dye (2 μ M) was diluted 1:50 in pure dimethyl sulfoxide (DMSO) in an amber 1.5-mL tube or covered with foil. Diluted 1X fluorescent dye was vortexed briefly and spun down and then stored protected

from light at room temperature until assembly (dye will precipitate if placed on ice).

LAMP Master Mix (15- μ L Reaction Volume) Setup

The RT-LAMP reaction mixes (see Supplemental Tables 1 and 2) included 2 primer mixes (the E1-LF + N2-LF or EN* mix for the COVID-19 test and the rActin mix as an internal control).

The following reagents were combined in 2 separate 1.5-mL microcentrifuge tubes in this order:

- 7.5- μ L NEB Colorimetric LAMP 2X Master
- 0.8- μ L 1X fluorescent dye
- 1.5- μ L 10X LAMP primers (EN*/rActin)
- 0.3- μ L Antarctic thermolabile UDG (2U/ μ L)
- 0.1- μ L dUTP (100 mM)
- 0.5- μ L guanidine hydrochloride (1.2 M)
- 0.75- μ L RNaseOUT (30 U/15 μ L reaction) or polyvinyl sulfonic acid (PVSA; 900 μ g/mL)

Mix thoroughly by vortexing at medium speed for 10 seconds, spin down, and protect from light until ready to plate.

Using a single or multichannel pipette, 11.45 μ L of EN* master mix was measured in aliquots into columns 2–4, 6–8, 10–12, 14–16, 18–20, and 22–24 of a 384-well optical plate. The rActin (11.45 μ L) master mix was measured in aliquots into columns 1, 5, 9, 13, 17, and 21. After sealing the plate with optical tape, we centrifuged at 4000 rpm for 5 minutes to remove bubbles and settle contents in wells. This plate was covered with aluminum foil and sealed in a zip-top bag and frozen at -20°C until ready to use.

Saliva Sample Preparation

Upon receipt, saliva was heat-inactivated at 95°C for 5 minutes and then placed on ice immediately for 3–10 minutes to chill. After cooling, the saliva was centrifuged at 5000 rpm or greater for 5 minutes to pellet debris. The supernatant was removed while carefully avoiding the pelleted material and transferred to a fresh 1.5-mL tube. This supernatant was then diluted 1:5X or 1:10X with 2.5-mM TCEP + 1-mM EDTA buffer prepared as per Rabe and Cepko's protocol²² and pipetted 10X to mix thoroughly, spun down, and stored on ice until ready to assay. When saliva samples were unable to be processed immediately, the diluents were frozen at -80°C for upwards of 2 weeks.

Running the Assay

On removal from the freezer, the plate was thawed at room temperature and protected from light 20 minutes before running the assay. Once thawed, the plate seal was removed, and 3.55 μ L of positive control was spiked into wells in columns 2, 6, 10, 14, 18, and 22; 3.55 μ L of no-template control (NTC; water) was spiked into wells in columns 3, 7, 11, 15, 19, and 23; then saliva samples diluted in 2.5-mM TCEP 1-mM EDTA pH 8.0 were spiked into wells in columns 1, 4, 5, 8, 9, 12, 13, 16, 17, 20, 21, and 24. After resealing the

plate with optical tape, it was vortexed briefly to mix, and this was followed by centrifugation at 4000 rpm for 5 minutes. The assay was performed on a thermal cycler or real-time PCR (qPCR) instrument with the following settings:

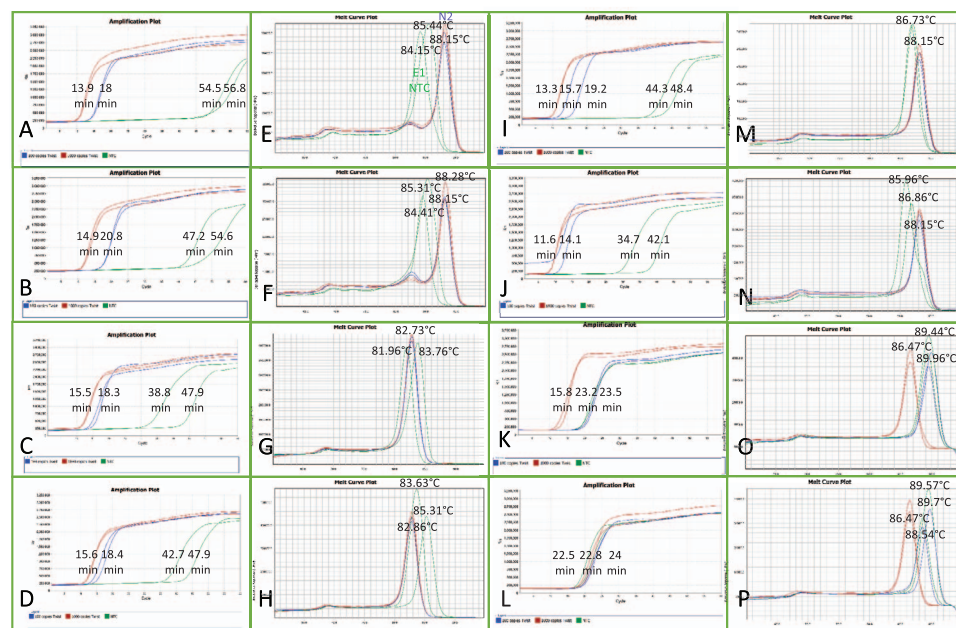
- 65°C for 30 minutes (data collection every 30 seconds for 80 cycles if using qPCR)
- 4°C for 5 minutes (or melt-curve analysis if using qPCR)

RESULTS

Initial testing was conducted using NEB WarmStart Colorimetric LAMP 2X Master Mix (DNA and RNA; M1800L) and WarmStart LAMP Kit (DNA and RNA; E1700L) using N-A and ORF1a primers from Dao Thi *et al.*,¹⁹ but due to low reproducibility and high false-positive rates with these primers, further assay development used E1 and N2 primer sets designed by Zhang *et al.*¹⁸ combined with 40-mM guanidine hydrochloride. The E1 and N2 primers with guanidine yielded dramatic improvements in sensitivity and specificity of the assay, demonstrating a limit of detection (LoD) of 10 copies per reaction using Twist Bioscience SARS-CoV-2 control RNA. However, despite these improvements, the assay still suffered sporadic nonspecific amplification in no-template controls. To eliminate carryover contamination as a potential cause of nonspecific amplification, Antarctic thermolabile UDG and dUTP were added to the reaction mixture. These components succeeded in quashing NTC amplification, but amplification in positive control reactions remained inconsistent.

Primer Mix Optimization

To address this shortcoming, both E1 and N2 primers were analyzed using Integrated DNA Technologies' OligoAnalyzer tool to determine whether cross-reactivity, such as primer-dimer or secondary structures in any of the primers might lead to inconsistent amplification as Meagher *et al.* suggest.²³ OligoAnalyzer results revealed that some primer pairs could form exceptionally strong secondary structures even at the LAMP incubation temperature of 65°C . In addition, it was found the E1-LF primer had a melting-temperature 20°C lower than any other primer in either the E1 or N2 primer sets, suggesting that it could inhibit amplification by out-competing the other primers during hybridization. As Khorosheva *et al.*²⁴ suggest in their research using digital real-time RT-LAMP, LAMP primers must hybridize to the template in a specific order to enable amplification to occur (F3/B3 \rightarrow FIP/BIP \rightarrow LF/LB); any other order terminates amplification abruptly. It is interesting that Khorosheva *et al.* also found in their research that although loop primers improve the speed of amplification in LAMP, these primers do not improve the reaction efficiency, such that a RT-LAMP reaction containing a single-loop primer can be as efficient as or more efficient than a reaction containing 2-loop primers. Ding *et al.*²⁵ also found that removing loop primers from a LAMP reaction can improve specificity and sensitivity. On the basis of the OligoAnalyzer data and the literature, all SARS-CoV-2 LAMP primer



Legend	
Panel	Description
A	Amplification Plot for RT-LAMP with N2-LF + E1-LF Primers Red curves are 1000 copies Twist Bioscience SARS-CoV-2 RNA control 2, blue curves are 100 copies Twist RNA, green curves are NTC
B	Amplification Plot for RT-LAMP with N2 + E1 Primers
C	Amplification Plot for RT-LAMP with E1 Primers
D	Amplification Plot for RT-LAMP with E1-LF Primers
E	Melt-curve plots RT-LAMP with N2-LF + E1-LF Primers (Blue and red curves correspond to N gene targets, green curves correspond to E gene amplification in NTC)
F	Melt-curve plots for RT-LAMP with N2 + E1 Primers
G	Melt-curve plots for RT-LAMP with E1 Primers
H	Melt-curve plots for RT-LAMP with E1-LF Primers
I	Amplification Plot for RT-LAMP with N2 Primers
J	Amplification Plot for RT-LAMP with N2-LF Primers
K	Amplification Plot for RT-LAMP with ORF1a Primers
L	Amplification Plot for RT-LAMP with ORF1a-LF Primers
M	Melt-curve plots for RT-LAMP with N2 Primers
N	Melt-curve plots for RT-LAMP with N2-LF Primers
O	Melt-curve plots for RT-LAMP with ORF1a Primers
P	Melt-curve plots for RT-LAMP with ORF1a-LF Primers

FIGURE 2

Primer optimization.

combinations were evaluated systematically to determine the optimum sets (see **Fig. 2**). The combined LAMP primer sets E1 and N2 that omitted the LF primers from both sets demonstrated the best specificity, sensitivity, and time to positive, so all further tests used this optimum primer combination.

Sample RNA Integrity Optimization

After primer optimization, sensitivity and specificity testing of direct saliva RT-LAMP was pursued. Initial sensitivity tests using mock positive saliva (saliva that was spiked with either Twist control RNA for SARS-CoV-2 or BEI SARS-CoV-2 cell lysate) gave inconsistent results, sometimes showing no amplification even with high RNA template inputs. These results indicated that active nucleases were present in the heat-inactivated saliva samples. To prevent further degradation of RNA that could result in false-negative tests, various RNase inhibitors were tested both in the sample preparation and the RT-LAMP master mix. The following RNase inhibitors were evaluated: NEB RNase inhibitor murine, Invitrogen SUPERase-In, and Invitrogen RNaseOUT Recombinant Ribonuclease Inhibitor. Only RNaseOUT at a final concentration of 30 units per reaction was found to consistently inhibit nuclease activity in direct saliva RT-LAMP at the optimum incubation temperature of 65°C. RNaseOUT performed equally well when used either in the master mix or applied to the saliva sample.

Sensitivity and specificity testing (**Fig. 3**) were conducted using negative saliva samples that were heat inactivated at 95°C for 5 minutes²¹ and diluted 1:10 in TCEP buffer before being

spiked with BEI SARS-CoV-2 isolate USA-WA1/2020 heat-inactivated cell lysate (NR-52286) (stock concentration = 375 000 copies/μL) to final concentrations of 1000 and 100 total genome copy equivalents per reaction. All reactions were run with 20 replicates. No template controls contained negative saliva diluted 1:10 in TCEP buffer.

Sensitivity and specificity for mock saliva tests containing 1000 copies or 100 copies of SARS-CoV-2 RNA were both 100% with combined E1-LF and N2-LF primer sets.

Assay Cost Reduction

Despite the dramatic improvement in reproducibility of the assay upon inclusion of RNaseOUT, it was felt that the cost of this nuclease inhibitor would be prohibitive especially in low-resource settings. To reduce cost and improve accessibility of the assay, chemical nuclease inhibitors were also investigated. A search of the literature^{20,21,26} revealed that the anionic polymer PVSA is an exceptionally potent and thermostable yet affordable RNase inhibitor when used in RT-qPCR, *in vitro* transcription (IVT), and RT-LAMP. Earl *et al.*²⁰ demonstrated that PVSA could improve messenger RNA integrity 5-fold when used in IVT at less than 1/1700 the cost of conventional ribonuclease inhibitors, and Smyraki *et al.*²¹ showed that PVSA at a final concentration of 45 μg/mL dramatically improved SARS-CoV-2 RNA stability in heat-inactivated nasopharyngeal swabs in their direct RT-qPCR assay. PVSA also lyses cells as Yu *et al.*²⁶ described, thereby improving RNA yield and potentially decreasing the LoD for direct detection from patient samples.

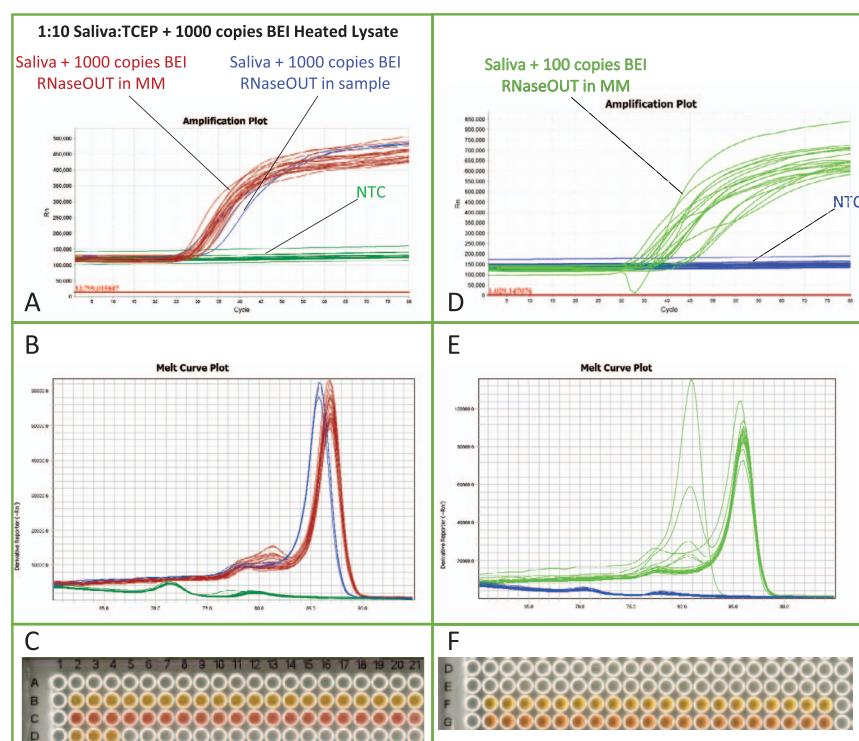


FIGURE 3

Sensitivity and specificity testing. MM, master mix.

Accuracy and LoD of Assay

LOD (copies/rxn)	Specificity	Sensitivity
1000	20/20	20/20
100	20/20	20/20

Legend

Panel	Description
A	Sensitivity test of 20 replicates: Wells B2-B21: Saliva + 1000 copies BEI Wells C2-C21: No template controls Wells D2-D4: Saliva + 1000 copies BEI + 30U RNaseOUT in sample
B	Melt-curve plots for assay in panel A. (Blue curves correspond to samples containing RNaseOUT, red curves correspond to LAMP master mix with RNaseOUT)
C	Colorimetric LAMP end-point readout of panel A
D	Sensitivity test of 20 replicates: Wells F2-F21: Saliva + 100 copies BEI Wells G2-G21: No template controls
E	Melt-curve plots for assay in panel D
F	Colorimetric LAMP end-point readout of panel D

Comparative sensitivity and specificity testing was done to evaluate the effectiveness of PVSA against RNaseOUT using the same conditions as previously described. These tests showed that PVSA outperformed RNaseOUT at preserving the integrity of RNA in saliva samples (Fig. 4). RT-LAMP master mix containing a final concentration of 45 $\mu\text{g/mL}$ PVSA demonstrated better reproducibility and quicker time to positive results than master mixes containing 30 U per reaction RNaseOUT. There were no false positives or false negatives, demonstrating the assay's exceptional robustness when testing saliva directly (Fig. 5).

After establishing that PVSA was at least as effective as RNaseOUT at preserving RNA integrity in saliva, sensitivity tests were conducted to determine the assay's LoD with PVSA. Mock saliva samples containing viral concentrations from 1000 copies per reaction to 10 copies per reaction were tested in triplicate. This test showed that the assay retained 100% specificity and sensitivity down to 50 copies per reaction, and 100% specificity and 33% sensitivity at 10 copies per reaction—2-fold lower than with RNaseOUT.

Stability Testing

After optimizing the master mix, stability testing was conducted to determine whether preplated master mix could withstand prolonged cold storage, thereby enabling scalability and dramatically reducing hands-on time for the assay. Three 384-well plates containing triplicate wells for internal controls,

positive controls, no template controls, and tests were prepared. Each plate was frozen at -20°C or -80°C for the following durations: 24 hours, 10 days, and 1 month. At each time point, 1 plate was removed and thawed at room temperature (Fig. 6) and then tested by adding and mixing the appropriate sample or control and run on an ABI QuantStudio 12K Flex qPCR instrument with the program described above.

DISCUSSION

During assay development several components and techniques were identified that proved essential to the reliability of the assay. There were also several shortcomings to the colorimetric LAMP assay, and the LAMP method in general was found to have a much steeper learning curve than conventional RT-qPCR. The following factors were critical to the assay's performance.

Screening LAMP Primers

Thorough *in silico* screening and bench testing of LAMP primer designs for nonspecificity are perhaps the most critical factor in developing a reliable assay, because LAMP reactions typically contain 4–12 primers or more in a reaction mixture. Consequently, nonspecific amplification is more probable with LAMP than conventional amplification methods, such as PCR, and false positives are a common hazard. Amplification of no-template controls were a common occurrence during the early stages of this assay's development, and inconsistent reproduc-

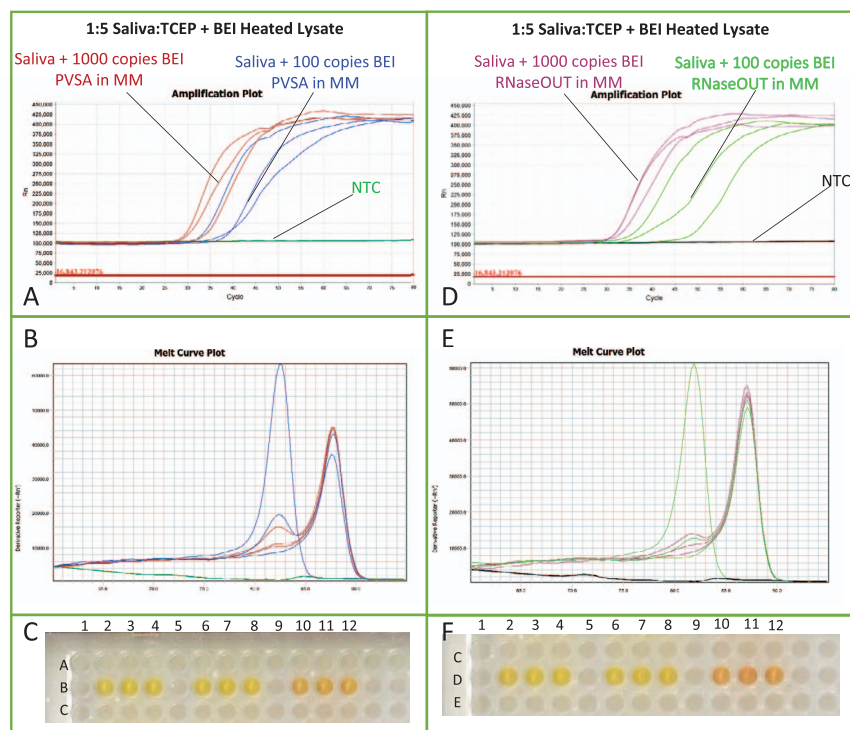


FIGURE 4

Comparative performance metrics of polyvinylsulfonic acid (PVSA) vs. RNaseOUT. MM, master mix.

Accuracy and LoD of Assay		
LOD (copies/rxn)	Specificity	Sensitivity
1000	3/3	3/3
100	3/3	3/3

Legend	
Panel	Description
A	Sensitivity test of 3 replicates for PVSA: Wells B2-B4: Saliva + 1000 copies BEI + 45 µg/mL PVSA Wells B6-B8-D4: Saliva + 100 copies BEI + 45 µg/mL PVSA Wells B10-B12: No template controls
B	Melt-curve plots for assay in panel A. (Blue curves correspond to samples containing 1000 copies BEI, red curves correspond to samples with 100 copies BEI)
C	Colorimetric LAMP end-point readout of panel A
D	Sensitivity test of 3 replicates for RNaseOUT: Wells D2-D4: Saliva + 1000 copies BEI Wells D6-D8: Saliva + 100 copies BEI Wells D10-D12: No template controls
E	Melt-curve plots for assay in panel D
F	Colorimetric LAMP end-point readout of panel D

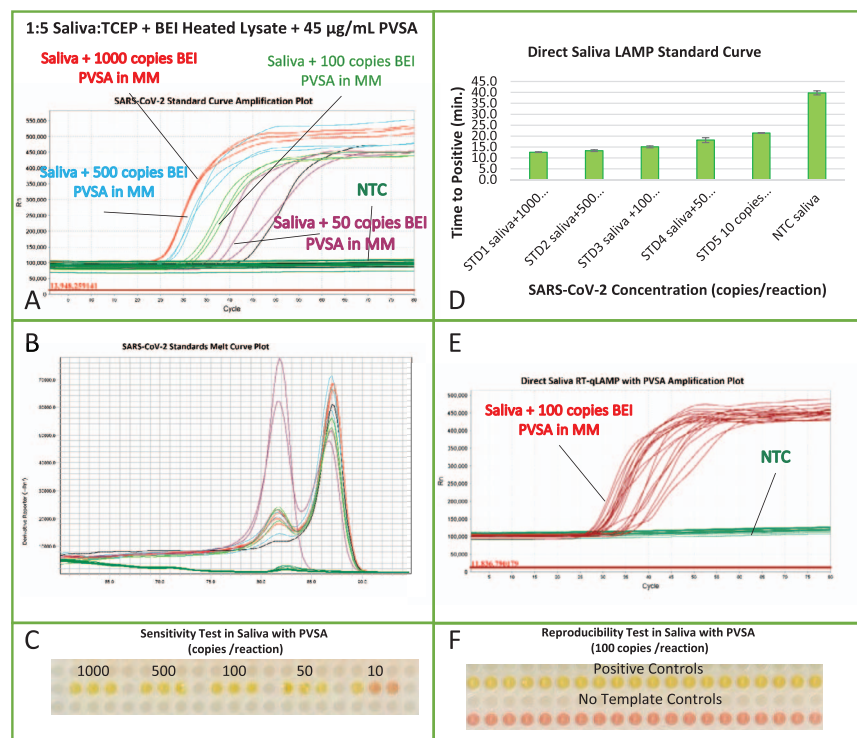


FIGURE 5

Sensitivity and specificity testing with polyvinylsulfonic acid (PVSA). MM, master mix; ST, standard.

Accuracy and LoD of Assay		
LOD (copies/rxn)	Specificity	Sensitivity
1000	3/3	3/3
500	3/3	3/3
100	3/3	3/3
50	3/3	3/3
10	3/3	1/3

Legend	
Panel	Description
A	Sensitivity test for direct saliva test with PVSA
B	Melt-curve plots for assay in panel A
C	Colorimetric LAMP end-point readout of panel A
D	Standard curve for direct saliva test with PVSA
E	Direct saliva test with 45 µg/mL PVSA, 20 replicates of 1000 copies BEI
F	Colorimetric LAMP end-point readout of panel E

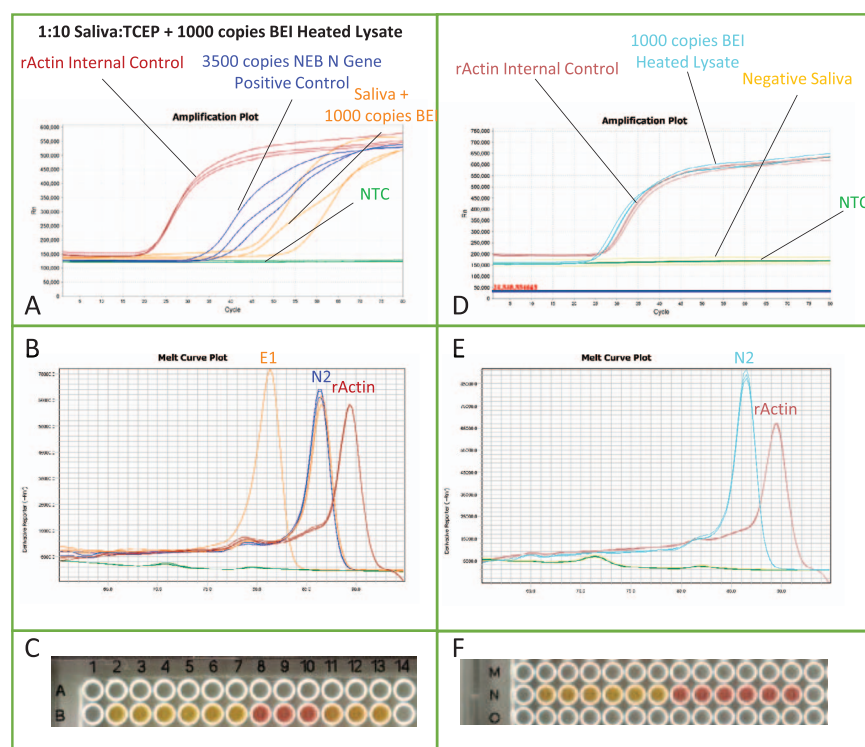


FIGURE 6

Stability testing. MM, master mix.

ibility of purified RNA positive controls even with high template concentrations strongly suggested that primer cross-reactivity was involved. In this assay, it was found that removing certain loop primers sacrificed some reaction speed but vastly improved specificity and reproducibility. This reliability carried over to direct saliva testing, indicating that the trade-off in speed for specificity was justified.

Primer specificity testing requires fluorometric validation to ensure amplification of true LAMP products. This is an essential stage of early RT-LAMP assay development and should not be overlooked. There are several methods now available for monitoring LAMP specificity, including molecular beacons,²⁷ fluorescent self-quenching LAMP probes (detection of amplification by release of quenching, DARQ²⁸), and quenching of unincorporated amplification signal reporters (QUASR²⁹), or nucleic acid-specific fluorescent dyes, such as SYTO-9. There are advantages of each chemistry, but for this assay SYTO-9 was chosen as the most economic and convenient solution, given that it was a component already available with the E1700 NEB WarmStart LAMP kit (DNA and RNA), and as others' research has shown,^{30–32} SYTO-9 is a reliable, noninhibitory fluorescent reporter in LAMP.

Reaction Additives

Through numerous productive conversations with Nathan Tanner of NEB and others of the Global LAMP Consortium, several reagents were identified that greatly improved the speed,

sensitivity, and reliability of the RT-LAMP assay. Among these key ingredients, guanidine hydrochloride¹⁸ was found to halve the time to results for the assay, enabling results in 30 minutes or less when 2 LAMP primer sets were used. It was also found that SYTO-9 fluorescent dye diluted to 0.05X in DMSO improved specificity and sensitivity of the quantitative RT-LAMP (RT-qLAMP) assays. This enhancement is consistent with Wang *et al.*³³ research that showed 7.5% DMSO could improve LAMP sensitivity and specificity.

Antarctic thermolabile UDG was another adjunct suggested by Nathan Tanner as a means of mitigating carryover contamination and reducing no-template amplification. The inclusion of this enzyme was found to be essential to preventing further false positives, which plagued early assay development.

Each of these components produced enormous improvements when tested using synthetic or purified RNA extracts. However, when direct saliva tests were conducted, the same results were not seen. This inconsistency suggested that RNA template degradation was occurring, and another additive was needed to protect the sample during the assay. As mentioned in the "Methods and Materials" section, various nuclease inhibitors were tested, but ultimately it was found that a very affordable anionic polymer (PVSA)^{20,21} was the best nuclease inhibitor for RT-LAMP, because it improved the LoD by 2-fold over the best conventional nuclease inhibitor tested, Invitrogen RNaseOUT, at negligible cost (Supplemental Table 3).

Legend	
Panel	Description
A	Stability Test 1: 10 days at -20C Wells B2-B4: HeLa Total RNA in rActin MM Wells B5-B7: 3500 copies NEB N gene plasmid Wells B8-B10: No template Controls Wells B11-B13: Saliva + 1000 copies BEI
B	Melt-curve plots for assay in panel A. (Blue curves correspond to N gene targets, red curves correspond to rActin internal control target)
C	Colorimetric LAMP end-point readout of panel A
D	Stability Test 1: 28 days at -20C Wells N2-N4: HeLa Total RNA in rActin MM Wells N5-N7: 1000 copies BEI heated lysate Wells N8-N10: No template controls Wells N11-N13: Saliva test (known negative)
E	Melt-curve plots for assay in panel D
F	Colorimetric LAMP end-point readout of panel D

Sample Treatment

Besides the various adjuncts and primer optimizations made to the standard NEB RT-LAMP reaction, it was found that saliva samples required specific treatment to be assayed directly. Several methods for direct saliva RT-LAMP were tested,^{5,6,22} some of which included proteinase K for inactivation of nucleases, but it was found that proteinase K remained partially active in the RT-LAMP reaction even after 95°C heat treatment and flash freezing on dry ice. In our tests, proteinase K often caused false-negative results in the RT-qLAMP assays. Residual proteinase K activity was also found to lead to false-positive results in no-template controls in the colorimetric assays, presumably due to hydrolysis of salivary proteins and other enzymes. For these reasons, proteinase K was omitted from future testing and nuclease inhibitors were focused on instead.

It was also determined during assay development that prolonged heat inactivation used in some protocols severely degraded viral RNA, causing false-negative results as others have reported.^{34–37} Consequently, we chose to use a modified version of Rabe and Cepko's protocol that involved heat treating saliva samples at 95°C for 5 minutes prior to dilution in 2.5-mM TCEP with 1-mM EDTA buffer. There are two major advantages to this technique. First, by heat inactivating saliva samples prior to the addition of other agents, this dramatically reduces exposure to potential biohazardous material for the tester. As Smyrlaki *et al.*²¹ confirmed by plaque assay, heat inactivation at 95°C for 5 minutes is sufficient to eliminate SARS-CoV-2 viral activity. The second major advantage of Rabe and Cepko's dilute TCEP solution was that it proved to consistently buffer saliva samples when diluted 5X–10X, regardless of the collection conditions. Mock tests conducted with saliva samples taken from known negative individuals who drank coffee or even smoked just prior to collection gave no false positives in any no-template controls by colorimetric detection, and all true positive spike-in controls amplified specifically by fluorimetry. This buffer greatly simplified the assay by eliminating the extra time and cost of additional enzymes and processing.

Future Developments

Herein, several improvements have been suggested for adapting a conventional RT-LAMP kit to be used for high-throughput direct saliva testing of SARS-CoV-2. However, several shortcomings of the assay were found, yet they remained unaddressed due to time and other constraints.

Foremost among these were recurrent ambiguous colorimetric results presented by the pH indicator dye used in the colorimetric LAMP mix, phenol red. Phenol red operates within a narrow pH range (6.2–8.238), which is convenient for the LAMP reaction, because a mildly buffered solution can demonstrate an observable color change after just a few minutes of amplification. Despite this, a great diversity of colors ranging from cerise to various shades of orange to bright yellow can occur during the reaction process. The pH of *any* reaction component will affect these changes in color. Consequently,

interpretation of colorimetric results with phenol red indicator can be very subjective. During our tests it was also found that certain additives could even prevent a color change from occurring (*e.g.*, inorganic pyrophosphatase), such that a known positive control sample that showed strong amplification by fluorescence appeared to remain negative by phenol red colorimetric detection. Had only a colorimetric reporter been used in these reactions, these could not have been identified as false negatives.

Fortunately, the current pandemic has spurred on unprecedented innovation in engineering, molecular biology, and chemistry, and several affordable alternatives to pH indicator dyes for indirect detection have been found that are adaptable to LAMP assays. These include the use of metal indicator dyes, such as hydroxynaphthol blue,³ eriochrome black T,³⁹ and calcein.⁴⁰ Numerous methods using dual-reporter fluorescent dyes have also been developed over the past few years for both indirect colorimetric end-point detection and direct fluorescent measurements, including leuco triphenylmethane dyes⁴¹ and various fluorescent intercalating dyes.^{30,32,42}

Another general fault of RT-LAMP assays, including fluorescent assays such as this one, is that these assays are semiquantitative at best and so cannot be used to accurately determine viral titer. However, recently developed hue-based LAMP assays such as the open-source smartphone-based eriochrome black T LAMP assay developed by Nguyen *et al.*³⁹ may soon enable point-of-care truly quantitative RT-LAMP for SARS-CoV-2 and other infectious diseases.

Assay cost and shelf stability are the two remaining hurdles that must be overcome to bring RT-LAMP out of the lab to enable regular affordable testing for all, and these challenges were only partially met by the assay we propose herein. Although we succeeded in reducing the cost per test to nearly half that of a conventional kit, \$1.80 per reaction is still far beyond what many people can afford to pay in developing countries. Most of the assay cost came from the proprietary enzymes used in the LAMP reaction mix. This cost could be greatly reduced by substitution of open-source enzymes as Kellner *et al.*³ demonstrated in their rapid beads-based SARS-CoV-2 RT-LAMP assay. Also, although we demonstrated that this assay remained stable when frozen for 1 month, we were unable to conduct shelf-stability tests for longer durations and did not test stability at higher temperatures, which is essential for a field-deployable assay for which refrigeration cannot be guaranteed. Lyophilization of reaction mixtures and stabilization with trehalose sugar can enable room temperature stability of LAMP master mixes,⁴³ thereby overcoming this obstacle too.

Fortunately, as Bektaş *et al.*⁴⁴ have shown with their accessible LAMP-enabled rapid test for SARS-CoV-2, these goals are reasonable and achievable.

CONCLUSIONS

The RT-LAMP assay presented here demonstrates that with some key modifications, a widely available commercial RT-

LAMP kit can be adapted for sensitive, robust, timely, and affordable direct detection of SARS-CoV-2 infection from saliva samples. This RT-LAMP formulation, which is stable for at least 4 weeks at -20°C , provides a low-cost, high-throughput method of testing for patient saliva samples directly and can be adapted for future epidemics. SARS-CoV-2 and other infectious diseases will remain a public health burden for the foreseeable future. RT-LAMP assays such as this can help alleviate that burden.

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